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**SYNERGISTIC INTERACTION OF HYPERTENSION AND DIABETES IN
PROMOTING KIDNEY INJURY AND THE ROLE OF
ENDOPLASMIC RETICULUM STRESS**

Zhen Wang,^{1,2} Jussara M. do Carmo,^{1,2} Nicola Aberdein,^{1,2} Xinchun Zhou,³

Jan M. Williams,^{1,4} Alexandre A. da Silva,⁵ John E. Hall.^{1,2}

¹Department of Physiology & Biophysics, ²Mississippi Center for Obesity Research,

³Department of Pathology, ⁴Department of Pharmacology and Toxicology, University of

Mississippi Medical Center, Jackson, Mississippi, USA; ⁵Barão de Mauá University

Center, Ribeirão Preto, São Paulo, Brazil.

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Corresponding Author:

Zhen Wang, Ph.D.
Department of Physiology and Biophysics
University of Mississippi Medical Center
2500 N. State St.
Jackson, MS 39216-4505
Phone: 601-984-1820
Fax: 601-984-1817
Email: zwang3@umc.edu

ABSTRACT

Diabetes mellitus and hypertension are major risk factors for chronic kidney injury, together accounting for >70% of end-stage renal disease. In this study, we assessed interactions of hypertension and diabetes in causing kidney dysfunction and injury and the role of endoplasmic reticulum (ER) stress. Hypertension was induced by aorta constriction (AC) between the renal arteries in 6-month old male Goto-Kakizaki (GK) type 2 diabetic and control Wistar rats. Fasting plasma glucose averaged 162 ± 11 and 87 ± 2 mg/dL in GK and Wistar rats, respectively. AC produced hypertension in the right kidney (above AC) and near normal blood pressure (BP) in the left kidney (below AC), with both kidneys exposed to the same levels of glucose, circulating hormones, and neural influences. After 8 wks of AC, BP above the AC (and in the right kidney) increased from 109 ± 1 to 152 ± 5 mmHg in GK rats and from 106 ± 4 to 141 ± 5 mmHg in Wistar rats. The diabetic-hypertensive right kidneys in GK-AC rats had much greater increases in albumin excretion and histological injury compared to left kidneys (diabetes only) of GK rats or right kidneys (hypertension only) of Wistar-AC rats. Marked increases in ER stress and oxidative stress indicators were observed in diabetic-hypertensive kidneys of GK-AC rats. Inhibition of ER stress with tauroursodeoxycholic acid (TUDCA) for 6 wks reduced BP (135 ± 4 vs 151 ± 4 mmHg), albumin excretion, ER and oxidative stress, glomerular injury, while increasing GFR in hypertensive-diabetic kidneys. These results suggest that diabetes and hypertension interact synergistically to promote kidney dysfunction and injury via ER stress.

Key words: glomerular filtration, type 2 diabetes, blood pressure, albumin excretion, oxidative stress, nephropathy

INTRODUCTION

The prevalence of diabetes mellitus continues to increase worldwide and in the United States nearly 10% of the population has diabetes. More than 90% of diabetic patients have type 2 diabetes and at least 70% are hypertensive¹⁻⁴. Hypertension in obese patients with type 2 diabetes is often difficult to control^{5, 6}. Surveys of responding diabetic patients indicate that 85% were taking antihypertensive drugs but only 36% had blood pressure (BP) controlled to the goal of <130/80 mmHg^{7, 8}.

The coexistence of diabetes and hypertension, especially when they are not adequately controlled, substantially increases the risk for onset and progression of chronic kidney disease (CKD) as well as cardiovascular morbidity and mortality. Although current therapeutic options may slow progression of diabetic-hypertensive nephropathy, many of these patients ultimately progress to end stage renal disease (ESRD)⁹⁻¹¹. Several pathological mechanisms, such as activation of renin-angiotensin-aldosterone system (RAAS), mechanical stretch, oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and apoptosis, have been postulated to contribute to diabetic-hypertensive nephropathy but the importance of these factors and their interactions are still unclear.

Chronic mechanical stresses associated with increases in BP may interact synergistically with hyperglycemia to cause kidney injury and some studies suggest that hypertension may be required for rapid progression of diabetic nephropathy¹². In two separate case reports, patients with long-standing diabetes and coexisting unilateral renal artery stenosis had no evidence of nephropathy in the kidney distal to the arterial stenosis, which was protected from hypertension, whereas the contralateral kidney

exposed to increased BP had severe nephropathy^{13, 14}. However, follow-up studies to replicate these results and to investigate potential mechanisms by which BP interacts with hyperglycemia to cause chronic kidney injury have not, to our knowledge, been reported.

Researchers have tried to replicate the hemodynamic effects of diabetes and hypertension in rodent models by administering high protein diets or by uninephrectomy, which both elevate glomerular hydrostatic pressure, or by superimposing diabetes on genetic models of hypertension¹⁵. For example, in transgenic rodents with excessively activated RAAS to induce hypertension there is accelerated development of diabetic nephropathy. However, it has been challenging in these studies to separate potential contributions of neural, hormonal, metabolic and other factors from BP effects on the kidneys. Furthermore, the cellular and molecular mechanisms by which hemodynamic effects may amplify hyperglycemia effects in causing kidney injury are still unknown.

Abnormal function of the ER, the specialized cytosolic organelle responsible for synthesis, packaging and assembly of secretory and membrane proteins, has attracted attention for its potential role in development of cellular injury. Stimuli that disrupt normal ER function may cause accumulation of unfolded or misfolded proteins, overwhelming the chaperones and causing ER stress¹⁶⁻¹⁹. Although ER stress may serve as a defense mechanism against external stresses, excessive ER stress eventually triggers pathological responses and has been implicated in obesity, diabetes and other cardiovascular diseases including hypertension²⁰. However, the role of ER stress and its relationship to renal dysfunction during development of diabetic-hypertensive nephropathy is poorly understood.

In this study, we used a rodent model of mild type 2 diabetes, the Goto-Kakizaki (GK) rat, combined with hypertension to test our hypothesis that diabetes and hypertension may interact synergistically to amplify oxidative stress and ER stress, and to promote progressive renal injury. The GK rat is a polygenic, non-obese model of type 2 diabetes with insulin resistance, deficient insulin production and mild diabetes²¹⁻²⁴. Renal injury in the GK rat is mild and slow to develop but can be amplified when hypertension is induced by administration of deoxycorticosterone acetate (DOCA) and a high salt diet²⁵. However, the importance of hemodynamic effects compared to other effects of mineralocorticoid receptor activation and high salt intake in causing kidney injury in previous studies is unclear.

To investigate the direct impact of increased BP in causing kidney injury when combined with diabetes, we developed a model that induces hypertension in one kidney of diabetic GK rats by aorta constriction (AC) between the renal arteries. The unique aspect of this model is that both kidneys are exposed to the same levels of hyperglycemia, circulating hormones, and neural influences but the left kidney below the AC has normal to slightly reduced BP while the right kidney above the AC is exposed to elevated BP.

To further understand the molecular mechanisms of diabetic-hypertensive nephropathy, we also used the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) to examine the role of ER stress during development of kidney injury caused by diabetes and hypertension. Our results suggest a synergistic interaction between hyperglycemia and hypertension that enhances ER stress and amplifies renal injury. Inhibition of ER stress markedly attenuates kidney injury in diabetic-hypertensive

nephropathy. These findings suggest that ER stress may be a therapeutic target to prevent development of diabetic-hypertensive nephropathy.

MATERIALS AND METHODS

Animals

The experimental procedures described in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Six-month old male GK and Wistar rats (Charles River Laboratories International, Inc., MA) were used in this study. The GK rat was developed as a model of type 2 diabetes by selective breeding of Wistar rats with the highest blood glucose levels during an oral glucose tolerance test over 35 generations²³.

Rats had free access to standard rat chow (Harlan Laboratories, Inc., IN) and were housed in individual cages maintained at $21\pm 2^{\circ}\text{C}$ and a 12:12-h light-dark cycle. Rats were randomly divided into six groups in this study: 1) GK rats with sham surgery (GK-Sham), 2) GK rats with aorta constriction surgery (GK-AC), 3) Wistar rats with aorta constriction surgery (Wistar-AC), 4) GK-AC rats with TUDCA treatment (GK-AC+TUDCA) 5) GK-Sham rats with TUDCA treatment (GK-Sham+TUDCA) and 6) GK-AC rats that received PBS vehicle treatment (GK-AC+Vehicle). The dose of TUDCA (200mg/kg/day, S.C., EMD Millipore, MA) was based on previous studies²⁶⁻²⁸ and our preliminary experiments showing effective inhibition of kidney tissue protein levels of the ER stress markers CHOP and GRP78, as assessed by western blot.

Surgical Procedures

A telemetric pressure transmitter device (model PA-C40, Data Sciences Int., MN) was implanted in the right common carotid artery and advanced into the aorta for 24-hr/day measurements of BP and HR in conscious rats as previously described²⁹. A 7-10 days recovery period after telemetry implantation was permitted prior to measuring baseline BP and HR for at least 5 additional days.

After stable baseline BP and HR measurements, the AC surgery was performed (see supplement for additional details). To constrict the aorta, a 22-gauge needle was placed next to the aorta between left and right renal arteries, and a suture was snugly tied around the needle and the aorta. Following ligation, the needle was removed and the muscles and skin were closed. The suture band between two renal arteries was carefully gauged to cause about 36% reduction of the outer diameter of aorta, a mild reduction in BP in the left kidney, and ~30 mmHg increase in BP of the right kidney after several days (**Figure 1A**). Rats were allowed 7 days to recover from AC surgery before the telemetric pressure transmitters were turned on again and BPs were recorded.

To determine BP gradient above and below the constriction, a femoral artery catheterization was also performed under isoflurane anesthesia on the last day of the experiment. After cannulation, the catheter was connected to Power Lab data acquisition system (ADInstruments, CO) to record BP. Simultaneously, BP was measured from the telemetry catheter implanted in the common carotid artery and advanced into the aortic arch, above the AC, and the pressure gradient across the AC was calculated. More detailed description of the procedures is provided in the online supplement.

Separate Kidney GFR and Urine Collection

Rats were anesthetized with 2% isoflurane and a midline abdominal incision was performed. A catheter was inserted into the femoral vein to infuse saline or 0.1% FITC-inulin (Sigma-Aldrich, MO) at the rate of 2.4 ml/h. To collect the urine from the right kidney, the right ureter was exposed and a catheter (RenaPulse RT 040, Braintree Scientific, Inc., MA) was inserted into the middle of the ureter. A flanged PE-40 size catheter was inserted into the bladder to collect urine from the left kidney. GFR of each kidney was measured over 2 hours using FITC-inulin clearance under isoflurane anesthesia as previously described³⁰.

Blood and Urine Biochemistry Measurements

Fasting blood glucose levels were measured using a glucose meter and strips (ReliOn Prime Blood Glucose Test Strips). Fasting plasma insulin and leptin concentrations were measured with ELISA (R&D Systems, MN and Crystal Chem Inc., IL respectively). 24 h total urine albumin levels were determined with ELISA (Crystal Chem Inc., IL) from urine collections for 72 hours in rat metabolic cages.

Western Blot for ER Stress Marker Protein

Proteins of renal cortex of left and right kidneys was isolated after homogenized in RIPA lysis buffer. Mouse polyclonal anti-CHOP (CCAAT-enhancer-binding protein homologous protein, 1:1000, Cell Signaling, MA) antibody was used to examine CHOP expression level.

4-HNE (4-Hydroxynonenal) Immunohistochemistry Staining

Both left and right kidneys from GK and Wistar rats were harvested and fixed in 10% formalin for 24 h and then embedded in paraffin and cut (5 µm) for 4-HNE staining. Sections were rehydrated, and antigens were unmasked in 10 mM sodium citrate, pH

6.0 heated at 95°C for 30 min, serum free protein blocker (Vector Laboratories, CA) was added, and then the sections were incubated with polyclonal anti-4-HNE antibody (Abcam, MA) diluted 1:500 overnight at 4°C in a humid chamber. After rinses with PBS, sections were incubated with secondary antibody provided in Vector ABC-HRP kit.

Renal Histology

Paraffin-embedded sections (5 µm) were prepared from kidneys fixed in 10% phosphate-buffered formalin. Periodic acid–Schiff (PAS) stain was used for analysis of renal morphology changes and Masson's trichrome stain was performed to observe fibrosis and collagen deposition in the kidney. Sections were scored in a blinded, semi-quantitative manner using an established scoring scale³¹. For each animal, at least 10 high power (400 x) fields were examined. The percentage of glomeruli that displayed basement membrane thickening, mesangial expansion, nodular sclerosis and global glomerulosclerosis were scored as follows: 0 = none, 1 = <25%, 2 = 25-50%, 3 = 50-75%, 4 ≥ 75%.

Transmission electron microscopy

Renal cortical tissues were cut into small pieces and rapidly immersed in tissue fixative buffer. After thin sectioned (70 nm in thickness) and applied on copper grids, the stained grid was loaded in a JOEL JEM1400 transmission electron microscopy (TEM) with an ANT camera system. At least 5 sections from each sample were examined under TEM. The entire sections were thoroughly viewed at low magnification (300×) for integrity and quality of stained tissues. Details of ultrastructural alterations were further investigated at high magnifications (20,000 x).

Statistical Analysis

Data are expressed as mean \pm SEM. A p value of <0.05 indicates significant difference. Significant differences between two groups were determined by Student's t -test. Significant differences between multiple groups at different time points or between left and right kidneys were determined by two-way ANOVA followed by Tukey's or Bonferroni's (for comparing left and right kidneys from the same animals) multiple comparison tests. Histologic scoring in kidneys was assessed by a pathologist who was blinded to the experimental protocols to avoid bias and the results were assessed using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Individual statistical analyses are described in the figure legends.

RESULTS

Anthropometric, Metabolic and Cardiovascular Characteristics of GK and Wistar Rats

Table 1 shows baseline characteristics of control Wistar and GK rats at 6 months old. Data are presented as average of all groups of GK or Wistar rats before AC or Sham surgery. GK rats were lighter and ate less food than Wistar control rats. However, fasting plasma glucose in GK was significantly increased compared to Wistar rats (162 ± 11 vs 87 ± 2 mg/dL) and accompanied with slightly higher fasting leptin and insulin levels. GK rats exhibited similar BP, 24-h urine output and 24-h urinary albumin excretion (UAE), but had reduced HR compared to Wistar control rats.

Impact of Aorta Constriction on Blood Pressure, Heart Rate, Body Weight, Food Intake and Blood Glucose in GK and Wistar Rats

AC rapidly increased MAP in GK and Wistar rats one week after surgery, from

109±1 to 134±6 mmHg in GK-AC rats and from 106±4 to 126±3 mmHg in Wistar-AC rats. After 8 weeks of AC, MAP averaged 152±5 and 141±5 mmHg in GK-AC and Wistar-AC rats, respectively (**Figure 1B**). In GK-Sham rats, MAP averaged 112±5 mmHg at baseline and did not change significantly during the 8 week study period. AC did not significantly alter heart rate, body weight, food intake or fasting blood glucose at 1,2,3,4,6, or 8 weeks in GK and Wistar rats (**Figures 1C-F**). There was no significant difference in BP gradient above and below AC in GK-AC and Wistar-AC rats (**Figure S1**).

Urinary Albumin Excretion

Total 24-h urines, including excretion from the left and right kidneys, were collected at baseline and 8 weeks after AC or sham surgery to assess renal function in GK and Wistar rats. Total urinary albumin excretion was not significantly different at baseline in GK and Wistar rats, averaging only 1.5-2.6 mg/24h (**Table 1**). After 8 weeks of AC, total urinary albumin excretion was significantly increased in GK-AC rats compared to baseline values. At 8 weeks AC, urinary albumin excretion in GK-AC rats increased to 59.3±17.2 mg/24h compared to only 11.0±6.0 mg/24h in Wistar-AC rats and 20.3±5.4 mg/24h in GK-Sham rats (**Figure 2A**).

Measurement of total 24-hr urinary albumin from both kidneys, however, does not differentiate which kidney is responsible for the large increase in albumin in GK-AC rats. Therefore, we also measured urinary albumin excretion from each kidney in Wistar-AC, GK-Sham, and GK-AC rats at 8 weeks after AC or sham surgery. **Figure 2B** shows that urinary albumin excretion was markedly increased in the right kidneys of GK-AC rats (28.2±8.9 µg/min, exposed to hyperglycemia and high BP) compared to the left kidneys

of the same animals (2.9 ± 0.8 $\mu\text{g}/\text{min}$, exposed only to hyperglycemia), the right kidneys from Wistar-AC rats (7.0 ± 3.3 $\mu\text{g}/\text{min}$ exposed only to high BP) and the right kidneys in GK-Sham rats (7.6 ± 2.2 $\mu\text{g}/\text{min}$, exposed only to hyperglycemia). These results indicate that increased urinary albumin in diabetic-hypertensive GK-AC rats mainly occurs in the hypertensive right kidneys exposed to increases in BP and hyperglycemia and suggest that coexistence of hypertension and diabetes may have synergistic effects to increase urinary albumin excretion.

Kidney Function

To evaluate interactions of high BP and diabetes on kidney function, kidney weight, urine output and GFR as well as were measured in left and right kidneys of GK and Wistar rats at 4 and 8 weeks after AC. In both GK-AC and Wistar-AC rats the right kidney weights were significantly greater than left kidney weights. Weight of right kidneys (exposed to hypertension plus diabetes) of GK-AC rats slightly increased from 1.7 ± 0.1 g at 4 weeks of AC to 2.0 ± 0.1 g after 8 weeks of AC. However, there were no significant differences in left kidney weight at 4 and 8 weeks of AC in GK-AC rats (**Figure 2C**). Urine output in the hypertensive-diabetic right kidneys was significantly increased compared to the left kidneys in GK rats at 4 and 8 weeks after AC (**Figure 2D**).

After 4 weeks of AC, GFR in the right kidneys of GK-AC rats was higher than in the left kidneys. However, after 8 weeks of AC, GFR in the right kidney declined substantially compared to the 4th week (from 1.1 ± 0.1 to 0.5 ± 0.1 ml/min/g, **Figure 2E**). GFR in the left kidney of GK-AC rats did not change significantly from 4 to 8 weeks after AC. These results indicate that combined hypertension and diabetes in the right kidney

causes an initial increase in GFR followed by a decline to normal within 4 weeks, associated with increased urinary albumin excretion.

Renal Glomerular Structural Changes

Renal morphological changes were assessed by PAS and Trichrome staining of kidney sections. Glomerular injury scores were assigned based on the severity of renal damage. As shown in **Figure 3A**, marked renal morphological changes, such as thickening of Bowman's capsule, expansion of glomerular mesangial matrix, increased cellularity, and tubular metaplasia formation in Bowman's capsule were observed in the right kidneys of GK-AC rats (**Figure 3A a-d**) compared to the right kidneys of diabetic GK-Sham rats, the left kidneys of GK-AC rats, and the right kidneys of Wistar-AC rats and (**Figure 3A e,f,g**) in PAS staining. Trichrome stain showed more collagen in Bowman's capsule and between the capillary loops of glomeruli in the right kidneys of GK-AC rats (**Figure 3A j**) compared to the right kidney of GK-Sham rats (**Figure 3A h**) and left kidney of GK-AC rats (**Figure 3A i**). A significantly higher renal injury score (**Figure 3B**) was found in the right kidneys of GK-AC rats compared to the left kidneys of GK-AC rats. However, kidneys from GK sham rats (exposed only to diabetes) or from Wistar-AC rats (exposed only to hypertension) did not show significant increases of glomerular injury scores.

Glomerular ultra-structural changes can also be observed by electron microscopy in the right kidneys of GK-AC rats compared to the left kidneys in the same animals or the right kidneys from GK-Sham rats. Disrupted glomerular ultrastructure, including detached endothelial layer in the glomerular capillary, thickening of the glomerular basement membrane, effacement of podocytes, and fusion of podocytes foot processes

(**Figure 3C**) were observed in the right kidneys of GK-AC rats.

ER stress and Oxidative Stress

To investigate the molecular pathways that mediate the synergistic effects of hypertension and diabetes on kidney structure and function, the ER stress marker, CHOP was measured by western blot of kidney cortex homogenates of GK-Sham, GK-AC and Wistar-AC rats. CHOP expression in the right kidneys of GK-AC rats exposed to high BP and high glucose was significantly greater than in the left kidneys exposed only to diabetes or the right kidneys of Wistar-AC rats exposed only to hypertension (**Figures 4A and B**).

We also performed immunohistochemistry for 4-HNE, an indicator of lipid peroxidation and oxidative stress, and found a much stronger 4-HNE staining in the right hypertensive kidneys of GK-AC rats compared to the left normotensive kidneys of the same diabetic rats or the right hypertensive kidneys of Wistar-AC rats after 8 weeks of AC (**Figure 4C and D**). Thus, increased 4-HNE staining and increased CHOP were observed only in kidneys exposed to the combination of hypertension and diabetes.

Treatment with ER Stress Inhibitor TUDCA Attenuates Hypertension

After 6 weeks of TUDCA treatment, there was a 52% reduction of CHOP in both left and right kidneys of GK-AC rats compared to untreated GK-AC rats (**Figure 5A**). TUDCA treatment also significantly attenuated the rise in BP in GK-AC rats (**Figure 5B**). At the end of TUDCA treatment, BP above the AC in GK-AC rats was 135 ± 4 mmHg compared to 151 ± 4 mmHg in vehicle treated GK-AC rats. TUDCA did not cause significant changes of BP in GK-Sham rats. We also found no significant changes in heart rate, food intake, body weight and blood glucose between vehicle and TUDCA

treated GK-AC rats (data shown in online supplement, **Figure S2 A,B,C**) indicating that the BP effects of TUDCA treatment were not due to reductions in food intake, body weight and blood glucose.

Treatment with TUDCA Improves Renal Function

TUDCA treatment significantly reduced 24-h total urinary albumin excretion to 15.1 ± 4.2 mg/24h in GK-AC rats compared to 59.2 ± 11.4 mg/24h in saline treated GK-AC rats (**Figure 5C**). Urinary albumin secretion was markedly reduced in the right kidneys of GK-AC rats treated with TUDCA compared to the right kidneys of GK-AC without treatment (5.6 ± 1.3 vs 30.9 ± 10.5 μ g/min, **Figure 5D**). GFR in the right kidneys of GK-AC rats treated with TUDCA was significantly higher than GFR in the right kidneys of GK-AC rats without treatment (0.9 ± 0.1 vs 0.6 ± 0.1 ml/min/g of kidney weight) (**Figure 5E**). We found no significant changes in GFR in the left kidneys of GK-AC treated with TUDCA or vehicle.

Treatment with TUDCA Improves Oxidative Stress and Preserves Normal Glomerular Structure

Chronic TUDCA treatment reduced oxidative stress in kidneys of GK-AC rats (**Figure 5F, upper right panel**). Positive 4-HNE staining area (**Figure S3**) was significantly reduced by approximately 76% in the right kidneys of GK-AC+TUDCA rats when compared to the right kidneys of GK-AC+Vehicle rats. TUDCA treatment also attenuated renal glomerular injury, as indicated by reduced thickness of glomerular basement membranes and attenuated expansion of mesangial matrix in the right kidney (**Figure 5F, lower right panel**). The overall kidney glomerular injury score in the right kidney of GK-AC+TUDCA rats was significantly reduced from 2.5 ± 0.3 to 1.2 ± 0.3 when

compared to the right kidneys of vehicle treated GK-AC rats (**Figure S4**).

DISCUSSION

An important finding of our study is that diabetes and hypertension have synergistic effects to promote renal dysfunction, albuminuria, ER stress, oxidative stress and glomerular injury in GK rats. This synergy was apparent even with mild hyperglycemia and moderate increases in BP, and significant renal injury developed rapidly over 8-weeks. Our results also demonstrated that inhibition of ER stress markedly attenuated renal dysfunction, albuminuria, oxidative stress and glomerular injury in kidneys exposed to hypertension and diabetes while producing mild reductions in BP in AC-induced hypertension.

We used GK rats for our studies since they develop mild spontaneous type 2 diabetes early in life, usually between 3-4 weeks of age, as a result of impaired ontogenetic development of islet cells, impaired insulin release following a glucose load, insulin resistance, hyperinsulinemia, and abnormal glucose metabolism similar to changes observed in humans with type 2 diabetes²¹⁻²³. GK rats at the age used in our studies do not have hypertension, glomerulosclerosis, tubulointerstitial fibrosis, or significant albuminuria and kidney dysfunction which are observed only at older ages (>18 months)³² when hypertension may also develop. These findings are similar to those observed in the preclinical phase of human diabetic nephropathy³³. Another attractive feature of GK rats is that they are not obese or hyperlipidemic, which may cause “lipotoxic” renal injury, making GK rats an excellent model to test the interaction of mild hyperglycemia and secondary injurious factors such as hypertension in contributing to CKD³⁴.

Previous studies have shown that renal injury in GK rats can be markedly

amplified when hypertension is induced by administration of DOCA and a high salt diet²⁵. However, the importance of increased BP compared to other effects of mineralocorticoid receptor activation and high salt intake in causing kidney injury in these studies is unclear, especially since there is evidence that mineralocorticoids may have BP-independent effects to cause renal injury and fibrosis^{35, 36}. Similar difficulties are encountered when interpreting results from studies in which diabetes-induced renal injury is accelerated by infusion of pressor agents such as AngII, transgenic overexpression of the RAAS, inhibition of nitric oxide synthesis or other experimental approaches to cause hypertension in diabetic animals^{37, 38}. In these types of studies it has also been challenging to separate potential contributions of multiple hormonal, metabolic, and neural changes induced by diabetes and/or the experimental method used to create hypertension from direct effects of increased BP and hyperglycemia on the kidneys.

In the present study, we induced hypertension in GK diabetic rats by AC between the two renal arteries. An important aspect of this model is that both kidneys were exposed to the same levels of hyperglycemia, circulating hormones, and neural influences but different perfusion pressures. This model therefore permitted us to compare the impact of differences in BP in the left and right kidneys of the same rats with or without high blood glucose. We also were able to investigate the effects on kidney function of diabetes alone in GK rats or hypertension alone in Wistar rats with AC and normal blood glucose.

Our results demonstrated that coexistence of hypertension and diabetes exerted synergistic effects to cause renal dysfunction and injury as reflected by increases in 24-

h urinary albumin excretion, ER stress, oxidative stress, histological injury of glomeruli, and slowly declining GFR. Although our study was not designed to provide detailed, quantitative histological assessment of kidney injury, the renal injury score based on PAS staining was consistently elevated only in the right kidneys of GK rats exposed to hypertension and diabetes. We also observed, using electron microscopy, glomerular ultra-structural changes including endothelial cell damage, increased basement membrane thickness thickening, podocyte effacement, and fusion of podocyte foot processes in the right kidney exposed to hypertension and diabetes in GK-AC rats. In the absence of hypertension, moderate hyperglycemia and hyperinsulinemia in GK rats were not associated with major kidney injury, severe albuminuria, glomerulosclerosis, ER stress, or kidney dysfunction. Eight weeks of moderate hypertension induced by AC between the renal arteries of Wistar rats also caused only modest albuminuria, ER stress and oxidative stress, and did not increase the renal injury score in the absence of diabetes.

Our results should not be interpreted as evidence that diabetes or hypertension alone cannot cause kidney dysfunction and injury. In fact, experimental studies have shown that chronic severe hyperglycemia can cause kidney injury, although renal lesions are often slow to develop. Also, in many experimental models and in humans with diabetes, chronic hyperglycemia may be associated with obesity and other metabolic or genetic abnormalities. For example in type 2 diabetes models such as Zucker fatty rats and db/db mice³⁹⁻⁴¹, the leptin receptor mutations cause severe obesity and hyperlipidemia, as well as hyperglycemia, that may contribute to kidney injury. Although chemical methods of inducing type 1 diabetes (e.g. streptozotocin and alloxan)

are not complicated by obesity, the amount of kidney injury is often mild despite severe chronic hyperglycemia and the agents used may themselves have toxic effects on the kidneys⁴².

Previous studies have shown that superimposition of hypertension on type 1 or type 2 diabetes produces much more severe kidney injury. Our results as well as clinical studies showing that tight BP control is at least as important as glycemic control in slowing progression of kidney disease support a major role for hemodynamic factors in the pathogenesis of diabetic kidney injury^{43, 44}. In fact, it has been suggested that increased BP may be a prerequisite for progression of diabetic nephropathy⁴⁵.

There is compelling evidence that hyperglycemia impairs normal autoregulation of GFR and renal blood flow^{46, 47}. To the extent that renal autoregulation is impaired, increases in systemic arterial pressure would be transmitted to the glomerular capillaries in diabetic kidneys to a greater degree than in normal kidneys. In our studies, there was initially a substantial rise in GFR that accompanied the moderate increase in BP of the right kidneys of diabetic GK-AC rats, suggesting impaired renal autoregulation. This initial glomerular hyperfiltration was followed by a decline in GFR to normal after only 8 weeks of diabetes and hypertension in the right kidneys of GK-AC rats. The rapid decrease of GFR in the hypertensive kidneys GK-AC rats is similar to the decline observed in diabetic patients who first undergo glomerular hyperfiltration followed by reductions of GFR to normal that are associated with nephron injury preceding a further decline of GFR to sub-normal levels as diabetic nephropathy progresses^{48, 49}. Therefore, one potential mechanism for the synergy of hypertension and diabetes to promote kidney injury is greater mechanical stress on the glomerular capillaries due to impaired

renal autoregulation, renal vasodilation, glomerular hyperfiltration, and greater transmission of increases in systemic arterial pressure to the glomerulus. However, there have been no previous studies, to our knowledge, that have assessed renal hemodynamics and autoregulation in diabetic GK rats exposed to hypertension as well as diabetes.

A hemodynamic perturbation could also alter sensing of extracellular glucose concentration and regulation of glucose uptake in glomerular cells. Mechanical stretch of human glomerular mesangial cells *in vitro* significantly upregulated expression of GLUT-1, a glucose transporter involved in cell glucose uptake. Glomerular GLUT-1 expression was also markedly increased in hypertensive Dahl salt-sensitive rats, compared to normotensive rats⁵⁰. Thus, Gnudi and colleagues⁵⁰ have suggested that mechanical stretching of glomerular cells may result in higher intracellular glucose concentration relative to actual ambient glucose. However, further studies are needed to determine the role of this hemodynamic-metabolic coupling in promoting kidney injury when diabetes and hypertension occur together.

Potential Role of ER Stress in Synergistic Effects of Diabetes-Hypertension Induced Kidney Injury

Although the underlying molecular mechanisms responsible for the synergy of hypertension and hyperglycemia to promote kidney injury are poorly understood, our results, suggest that ER stress may play an important role. The ER stress marker CHOP was markedly increased in the right kidneys of GK-AC rats exposed to increased BP and hyperglycemia, whereas kidneys subjected to chronic hypertension or diabetes alone had minimal increases in CHOP. Also, chronic administration of the ER stress

inhibitor TUDCA not only reduced CHOP but also markedly decreased albumin excretion and the glomerular injury score while attenuating the decline in GFR in kidneys of GK-AC rats exposed to hypertension and diabetes. In fact, TUDCA administration essentially normalized albumin excretion and the renal injury score of diabetic-hypertensive kidneys in GK-AC rats. To our knowledge this is the first report showing that inhibition of ER stress attenuates kidney dysfunction and injury induced by diabetes and hypertension together.

TUDCA administration also attenuated the rise in systemic arterial pressure in GK-AC rats. The mechanisms responsible for the BP lowering effects of TUDCA are unclear but could be related, in part, to attenuation of renal injury. However, TUDCA was administered systemically in our studies and we cannot ascertain the contribution of the direct effects on the kidneys compared to extrarenal actions that may have contributed to reductions in BP in GK-AC rats.

Inhibition of ER stress with TUDCA has been reported to reduce blood glucose in type 2 diabetic mice⁵¹. In our study, however, TUDCA treatment for 6 weeks did not significantly alter blood glucose in GK-AC rats. Therefore, reductions in blood glucose cannot explain the beneficial effects of TUDCA on kidney structure and function in GK-AC rats.

The cellular mechanisms that lead to ER stress in kidneys subjected to diabetes and hypertension are unclear but may be related, in part, to increased mitochondrial ROS production. Previous studies have suggested that mitochondrial metabolic overload results in increased cellular oxidative and ER stress, which leads to the activation of the unfolded protein response (UPR)^{52, 53}. During its early phase, the UPR

either refolds the accumulated unfolded proteins or degrades them via the ubiquitin-proteasome pathway⁵⁴. When the unfolded protein and cellular damage exceed a threshold and the chronic cell stress is not relieved, proapoptotic responses are initiated and cell death ultimately ensues^{55, 56}. Although our studies were not designed to assess the role of ROS in promoting kidney injury, we found that 4-HNE, an indicator of lipid peroxidation and oxidative stress, was markedly upregulated in hypertensive kidneys of GK-AC rats compared to normotensive kidneys of the same diabetic rats or hypertensive kidneys of Wistar-AC rats after 8 weeks of AC. However, further studies will be needed to determine whether crosstalk between ER and the mitochondria may account for excess ROS and kidney dysfunction and injury when diabetes and hypertension coexist.

Perspectives

Our results indicate that hypertension and diabetes interact synergistically to promote renal dysfunction, albuminuria, ER stress, oxidative stress and glomerular injury in a rodent model of type 2 diabetes. This synergy occurs even with mild hyperglycemia and hypertension. We also found that inhibition of ER stress markedly attenuated dysfunction and injury in kidneys exposed to hypertension and diabetes. The molecular mechanisms by which mechanical forces interact with high glucose levels to induce ER stress and renal injury warrant further investigation, especially since type 2 diabetes is often associated with hypertension and metabolic abnormalities that are difficult to control. An important implication of these studies is that simultaneous tight control of hypertension and hyperglycemia may be required to slow, or prevent,

progression of diabetic nephropathy. Inhibition of ER stress may be a new therapeutic strategy for diabetic nephropathy.

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Disclosures

None.

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NOVELTY AND SIGNIFICANCE

What Is New?

- Moderate increases in blood pressure and mild hyperglycemia interact synergistically to promote renal dysfunction, albuminuria, ER stress, oxidative stress and glomerular injury in a rodent model of type 2 diabetes.
- Pharmacological inhibition of ER stress attenuates increases in blood pressure and kidney injury in hypertensive-diabetic nephropathy

What Is Relevant?

- Hypertension and metabolic abnormalities are often difficult to control in patients with type 2 diabetes and current therapeutic options for diabetic-hypertensive nephropathy only slow rather than halt the progression of chronic renal disease.
- Our study illustrates the synergistic interaction of mild diabetes and hypertension in promoting kidney injury and highlights the importance of tight control of blood pressure and glycemia in arresting kidney injury.
- Our study provides a better understanding of the pathogenesis of diabetic-hypertensive renal injury and may provide novel therapeutic strategies that, when combined with current therapeutic interventions, help prevent progression to ESRD in diabetic patients.

Summary

We created a new experimental model to investigate the mechanisms of kidney injury in type 2 diabetes. Our findings indicate that mild hyperglycemia and hypertension interact synergistically to promote renal dysfunction, albuminuria, ER stress, oxidative stress and glomerular injury. We also found that inhibition of ER stress markedly

attenuated dysfunction and injury in kidneys exposed to hypertension and diabetes, suggesting a new therapeutic strategy for hypertensive-diabetic nephropathy.

FIGURE LEGENDS

Figure 1. A, Aortic constriction between the renal arteries induced higher BP in right kidney and normal or slightly reduced BP in the left kidney. After 8 wks of AC, mean arterial pressure above and below the constriction in GK-AC rat were significantly different. (n=9 *, $P<0.05$, compared to left kidneys). **B**, Mean arterial pressure measured by telemetry above the AC, **C**, Heart rate, **D**, Daily food intake, **E**, Body weight and **F**, Blood glucose measured at baseline, 1, 2, 3, 4, and 8 weeks after AC or sham surgery in GK and Wistar rats. (n=6 in Wistar-AC and GK-Sham groups, n=9 in GK-AC group; *, $P<0.05$ compared to GK-Sham rats at 8th week after AC or sham surgery and †, $P<0.05$ compared to Wistar-AC rats at 8th week after AC or sham surgery by two-way ANOVA followed by Tukey's post hoc test)

Figure 2. A, 24-h total urinary albumin secretion at baseline and 8 weeks after AC or sham surgery (n=6 in all groups; *, $P<0.05$ compared to left kidneys of GK-AC rats; †, $P<0.05$ compared to Wistar-AC rats at 8 weeks after AC surgery; #, $P<0.05$ compared to GK-Sham rats at 8 weeks after sham surgery by two-way ANOVA followed by Bonferroni's test) **B**, Urinary albumin excretion from left and right kidneys in Wistar-AC, GK-AC and GK-sham rats after 8 weeks after AC or sham surgery (n=6 in all groups; *, $P<0.05$ compared to left kidneys in GK-AC rats; †, $P<0.05$ compared to right kidneys of Wistar-AC rats; #, $P<0.05$ compared to right kidneys of GK-Sham rats by two-way

ANOVA followed by Bonferroni's test) **C**, Kidney weight, **D**, Urine output and **E**, GFR from left and right kidneys in Wistar-AC, GK-AC and GK-Sham rats at 4 and 8 weeks after AC or sham surgery (n=6 in all groups; *, $P<0.05$, compared to left kidneys within the same group of rats; †, $P<0.05$ compared to right kidneys of Wistar-AC rats at 8 weeks after AC surgery; #, $P<0.05$ compared to right kidneys of GK-AC rats at 4 weeks after AC surgery by two-way ANOVA followed by Bonferroni's test)

Figure 3. A, PAS and Trichrome staining of kidney slices from Wistar-AC, GK-AC and GK-Sham rats after 8 weeks of AC or sham surgery. PAS staining showed glomerulopathy (arrow) in diabetic-hypertensive right kidneys in GK-AC rats (**a**, mesangial expansion; **b**, increased thickness of Bowman's capsule; **c**, glomerulosclerosis; **d**, tubular metaplasia formation in Bowman's capsule) compared to **e**, right kidney in GK-Sham rats, **f**, left kidney in GK-AC rats and **g**, right kidney in Wistar-AC rats. Masson's trichrome staining showed collagen staining in **h**, right kidney of GK-Sham rats; **i**, left kidney of GK-AC rats and **j**, right kidney of GK-AC rats. Increased blue staining can be observed in glomeruli of the right kidneys of GK-AC rats (empty arrow). Scale bars (40 μm in e,f,g; 20 μm in a-d and h-j) **B**, Renal injury was scored based on the renal morphological changes at 8 weeks of AC or sham surgery in GK and Wistar rats. (n=6 in GK-Sham, n=7 in Wistar-AC and n=10 in GK-AC rats. *, $P<0.05$, compared to other groups by Kruskal nonparametric test followed by Dunn's multiple comparisons test) **C**, Representative images of transmission electron microscopy scans from **a**, the right kidneys of GK-Sham rats; **b**, left kidneys of GK-AC rats; and **c**, right kidneys of GK-AC rat. Arrow indicates endothelial cells, asterisk

indicates glomerular basement membrane, and empty arrow head indicates the foot process of podocytes.

Figure 4. **A**, Immunoblot examples of renal cortex CHOP expression in Wistar-AC, GK-AC and GK-sham rats at 8th week of AC. **B**, optical density analysis of CHOP expression. **C**, Representative immunohistochemistry staining of 4-HNE in left and right kidney slices of Wistar-AC, GK-Sham, and GK-AC rats after 8 weeks of AC or sham surgery (Scale bar, 20 μ m) and **D**, Pixels area of positive staining of 4-HNE. (n=5 in all groups; *, $P<0.05$, compared to other groups by two-way ANOVA followed by Bonferroni's multiple comparisons test)

Figure 5. **A**, Optical density analysis of renal cortex CHOP expression in GK-AC and GK-AC+TUDCA rats at 8 weeks of AC (n=5 in all groups *, $P<0.05$, compared to left kidneys from GK-AC rats and †, $P<0.05$, compared to right kidneys from GK-AC rats by t-test). **Insert**, immunoblot of CHOP expression. **B**, Mean arterial pressure change in GK-AC rats with TUDCA (n=7) or Vehicle (n=6) treatment and GK-Sham rats with TUDCA treatment (n=5) at baseline, 2, 3,4,6 and 8 weeks after AC or sham surgery. (*, $P<0.05$ compared to GK-Sham+TUDCA; †, $P<0.05$ compared to GK-AC+Vehicle at 8 weeks after AC or sham surgery by two-way ANOVA followed by Tukey's post hoc test) **C**, 24-h urinary albumin excretion (n=6 in all groups; #, $P<0.05$ compared to GK-AC+Vehicle rats by one-way ANOVA followed by Tukey's test); **D**, Individual left and right kidney urinary albumin excretion and **E**, individual kidney GFR measurements in GK-AC rats with or without TUDCA treatment (n=6) and GK-Sham rats with TUDCA

treatment (n=5) at 8 weeks of AC or sham surgery. (*, $P<0.05$, compared to left kidneys within the same group of rats; †, $P<0.05$ compared to right kidneys of GK-Sham+TUDCA rats; #, $P<0.05$ compared to right kidneys of GK-AC+Vehicle rats by two-way ANOVA followed by Bonferroni's test). **F**, Representative images of IHC staining for 4-HNE (**top**) and PAS staining (**bottom**) of kidney slices from right kidneys of GK-Sham+TUDCA, GK-AC+Vehicle and GK-AC+TUDCA rats at 8th week of AC (Scale bar, 20 μ m). Attenuated glomerulopathy was observed in diabetic-hypertensive right kidneys with 6 weeks TUDCA treatment.

Table 1. Baseline parameters of 6-month old GK and Wistar rats

Parameters	Wistar (n=10)	GK (n=10)
Body Weight (g)	649.4 ± 10.3	432.1 ± 10.1 *
Food Intake (g/24 h)	24.4 ± 1.1	19.1 ± 0.6 *
Normalized Food Intake (mg/24 h/g of BW)	36.0 ± 2.2	44.0 ± 1.6 *
Urine Output (ml/24 h)	47.5 ± 4.0	37.3 ± 8.0
Normalized Urine Output (ml/24 h/g of BW)	0.07 ± 0.01	0.10 ± 0.03
Urinary Albumin Excretion (mg/24 h)	1.5 ± 0.6	2.6 ± 0.6
Fasting Glucose (mg/dL)	87 ± 2	162 ± 11 *
Fasting Insulin (ng/mL)	0.9 ± 0.2	1.8 ± 0.2 *
Fasting Leptin (ng/mL)	2.0 ± 0.3	3.7 ± 0.3 *
Mean Arterial Pressure (mmHg)	106 ± 3	107 ± 2
Systolic Blood Pressure (mmHg)	94 ± 3	95 ± 3
Diastolic Blood Pressure (mmHg)	114 ± 3	119 ± 2
Heart rate (beats/min)	341 ± 4	304 ± 5 *

* $P < 0.05$ between the groups